Exhibit 4

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 41, Issue of October 10, pp. 40317–40323, 2003
Printed in U.S.A.

Inhibition of the Norepinephrine Transporter by the Venom Peptide χ-MrIA

SITE OF ACTION, Na^+ DEPENDENCE, AND STRUCTURE-ACTIVITY RELATIONSHIP*

Received for publication, December 20, 2002, and in revised form, July 2, 2003
Published, JBC Papers in Press, July 28, 2003, DOI 10.1074/jbc.M213030200

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χ-Conopeptide MrIA (χ-MrIA) is a 13-residue peptide contained in the venom of the predatory marine snail *Conus marmoreus* that has been found to inhibit the norepinephrine transporter (NET). We investigated whether χ-MrIA targeted the other members of the monoamine transporter family and found no effect of the peptide (100 μM) on the activity of the dopamine transporter and the serotonin transporter, indicating a high specificity of action. The binding of the NET inhibitors, [^{3}H]nisoxetine and [^{3}H]mazindol, to the expressed rat and human NET was inhibited by χ-MrIA with the conopeptide displaying a slight preference toward the rat isoform. For both radioligands, saturation binding studies showed that the inhibition by χ-MrIA was competitive in nature. It has previously been demonstrated that χ-MrIA does not compete with norepinephrine, unlike classically described NET inhibitors such as nisoxetine and mazindol that do. This pattern of behavior implies that the binding site for χ-MrIA on the NET overlaps the antidepressant binding site and is wholly distinct from the substrate binding site. The inhibitory effect of χ-MrIA was found to be dependent on Na^+ with the conopeptide becoming a less effective blocker of [^{3}H]norepinephrine by the NET under the conditions of reduced extracellular Na^+. In this respect, χ-MrIA is similar to the antidepressant inhibitors of the NET. The structure-activity relationship of χ-MrIA was investigated by alanine scanning. Four residues in the first cysteine-bracketed loop of χ-MrIA and a His in loop 2 played a dominant role in the interaction between χ-MrIA and the NET. Hα chemical shift comparisons indicated that side-chain interactions at these key positions were structurally perturbed by the replacement of Gly-6. From these data, we present a model of the structure of χ-MrIA that shows the relative orientation of the key binding residues. This model provides a new molecular caliper for probing the structure of the NET.

Because of its poor lipid solubility and degree of ionization at physiological pH, norepinephrine crosses cell membranes poorly by diffusion (1) and so relies on the operation of the norepinephrine transporter (NET)[1] for uptake into cells. Clearance by this integral membrane protein constitutes the major mechanism for the termination of action of this neurotransmitter at noradrenergic synapses (2), and disturbances in the functioning of the NET are associated with pathological states including depression (3), congestive heart failure (4), and orthostatic intolerance, and tachycardia (5). Known inhibitors of the NET include antidepressants (*e.g.* desipramine and nisoxetine), the appetite suppressant mazindol, and the abused drug cocaine (for review, see Ref. 6). The NET, together with the dopamine transporter (DAT) and the serotonin transporter (SERT), forms a family of Na^+- and Cl^--dependent monoamine transporters.

A novel peptidic NET inhibitor, χ-MrIA, has been identified in cone snail venom (7). Cone snails use a venom containing a mixture of bioactive peptides ("conopeptides") to capture their prey, and these are known to target an array of voltage-sensitive ion channels, ligand-gated ion channels, and G protein-coupled receptors (for review, see Ref. 8). Intrathecal injection of χ-MrIA has been found to be analgesic in hot plate and neuropathic pain models (9, 10). The inhibition of [^{3}H]norepinephrine uptake by the NET caused by χ-MrIA was found to be non-competitive, reducing the maximum rate of transport and not affecting the affinity of the transporter for substrate (7). The non-competitive mode of action of χ-MrIA distinguishes it from the majority of the classically described inhibitors of the NET that act in a competitive fashion. In this study, we explored the interaction of χ-MrIA with the monoamine transporters to gain an insight into the selectivity, Na^+ dependence, site of action, and structure-activity relationship of the conopeptide.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—χ-MrIA and the singly substituted analogs, [N1A]MrIA, [G2A]MrIA, [V3A]MrIA, [G6A]MrIA, [Y7A]MrIA, [K8A]MrIA, [L9A]MrIA, [H11A]MrIA, [O12A]MrIA, [Y7F]MrIA, and [K8R]MrIA, were synthesized. The chain assembly of the peptides was performed on a manual shaker system using HBTU activation protocols (11) to couple the Fmoc-protected amino acid to the resin. The Fmoc-protecting group was removed using 50% piperidine in dimethylformamide, and dimethylformamide was used as both the coupling solvent and for flow washes throughout the cycle. The progress of the assembly was monitored by quantitative ninhydrin monitoring (12). Peptide

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The atomic coordinates and structure factors (code 1IEO) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[1] The abbreviations used are: NET, norepinephrine transporter; DAT, dopamine transporter; SERT, serotonin transporter; 5-HT, 5-hydroxytryptamine (serotonin); NOESY, Nuclear Overhauser enhancement spectroscopy; TOCSY, total correlated spectroscopy; HBTU, *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uranium hexafluorophosphate); Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; ANOVA, analysis of variance.

was deprotected and cleaved from the resin by stirring at room temperature in trifluoroacetic acid:H_2O:triisopropylsilane:ethanedithiol (90:5:2.5:2.5) for 2–3 h. Cold diethyl ether was then added to the mixture, and the peptide precipitated out. The precipitate was collected by centrifugation and subsequently washed with further cold diethyl ether to remove scavengers. The final product was dissolved in 50% aqueous acetonitrile and lyophilized to yield a fluffy white solid. The crude, reduced peptide was examined by reverse phase high performance liquid chromatography for purity, and the correct molecular weight was confirmed by electrospray mass spectrometry. Pure, reduced peptides were oxidized, and the major peak was purified to >95% purity and characterized by high performance liquid chromatography prior to further use.

Cellular Uptake of [^{3}H]Monoamines—COS-1 cells (ATCC, Manassas, VA) were grown in 24-well plates (Falcon, BC Biosciences) containing Dulbecco's modified Eagle medium (Invitrogen) and 10% fetal bovine serum (Invitrogen) at 37 °C in 5% CO_2. Upon reaching ~85% confluency, the cells were transiently transfected with plasmid DNA encoding the human NET (13), the rat NET (14), the human DAT (15), or the human SERT (16). Transfections were performed using LipofectAMINE 2000 reagent (Invitrogen) following the manufacturer's protocol using 800 ng of DNA/well. Assays measuring the cellular accumulation of the transporters' respective [^{3}H]monoamine substrates were performed 24 h after transfection at room temperature in duplicate. The culture medium was removed, and the cells were washed three times with 500 μl of transport buffer containing 125 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 25 mM HEPES, 5.55 mM D-(+)-glucose, 1.02 mM ascorbic acid, 10 μM U-0521 (to inhibit catechol-*O*-methyl transferase), and 100 μM pargyline (to inhibit monoamine oxidase), pH 7.4. In experiments examining the Na^+ dependence of the NET inhibitors, the concentration of NaCl used in the transport buffer ranged from 25 to 125 mM with appropriate concentrations of LiCl added to retain equal osmolarity. Inhibitor drugs were preincubated with the cells for 15 min before the addition of 100 nM [^{3}H]monoamine substrate (supplemented with unlabeled substrate as required). The final volume was 250 μl. Nonspecific uptake of [^{3}H]norepinephrine by NET-transfected cells was defined by the accumulation occurring in the presence of 10^{-4} M desipramine. Imipramine (10^{-6} M) and GBR-12909 (10^{-6} M) were used to determine the amount of nonspecific uptake of [^{3}H]serotonin by SERT-transfected cells and [^{3}H]dopamine uptake by DAT-transfected cells, respectively. Transfected cells were exposed to [^{3}H]monoamine substrate for either 8 min (rat NET, human SERT) or 15 min (human NET, human DAT). The selection of these incubation times was based on the results of pilot studies that showed that the relationship between uptake and time was linear over these periods (data not shown). The solution containing unaccumulated ^{3}H-substrate was then rapidly removed, and the cells were washed three times with 1 ml of ice-cold phosphate-buffered saline. The cells were lysed with 0.1% Triton X-100 in 10 mM Tris·HCl, pH 7.5, for 60 min at room temperature with gentle shaking. The level of radioactivity of the cell lysate was determined by liquid scintillation counting.

Membrane Preparation—COS-7 cells (ECACC, Salisbury, Wiltshire, United Kingdom) were grown in 150-mm dishes and transiently transfected with 15 μg of plasmid DNA encoding the rat NET using the same method described for the uptake experiments. Membranes were prepared from cells 48 h after transfection for use in radioligand binding experiments. After washing the cells with warm phosphate-buffered saline, ice-cold TEM buffer (10 mM Tris·HCl, 1.4 mM EGTA, 12.5 mM $MgCl_2$, pH 7.5) was added and the cells were scraped from the dish. Cells were then homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and centrifuged at 1000 × *g* for 5 min at 4 °C to remove cellular debris then at 15,000 × *g* for 45 min at 4 °C. Pellets were washed with TEM buffer and recentrifuged. The resulting pellet was resuspended in TEM buffer containing 10% glycerol. Rat brain homogenates were prepared as described previously (17). Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL) following the manufacturer's protocol. Aliquots of membrane were stored at −80 °C until use.

Radioligand Binding Assays—Binding reactions were set up in triplicate wells of 96-well plates. Membranes from COS-7 cells transfected with the rat or human NET (6 μg of protein/well) were incubated with either [^{3}H]nisoxetine (4.3 nM) or [^{3}H]mazindol (4 nM) in the absence or presence of χ-MrIA or one of its analogs (1 nM–100 μM) in buffer containing 20 mM Tris·HCl, 75 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% bovine serum albumin, pH 7.4, for 1 h at room temperature. The final assay volume was 150 μl. The amount of nonspecific binding was determined by the inclusion of desipramine (100 μM) in the reaction. Bound and free radioactivity were separated by rapid vacuum filtration onto GF/B filters (Wallac, Boston, MA) pretreated with 0.6% polyethylenimine. Filter mats were washed three times with ice-cold buffer containing 25 mM HEPES, 125 mM NaCl, pH 7.4, and allowed to dry. Filter-retained radioactivity was quantified by liquid scintillation counting. For saturation analysis experiments, the binding reactions contained 6 μg of membrane protein from rat NET-transfected COS-7 cells, either [^{3}H]nisoxetine (4–100 nM) or [^{3}H]mazindol (5–86 nM) and χ-MrIA (0, 2, or 20 μM). In other experiments, rat brain homogenates (equivalent to 20 μg of protein/well) and [^{3}H]nisoxetine (4.3 nM) were incubated together in the absence and presence of unlabeled nisoxetine, desipramine, or χ-MrIA (1 pM–100 μM) in buffer containing 50 mM Tris·HCl, 300 mM NaCl, 5 mM KCl, pH 7.4, for 1 h at room temperature. The reactions were filtered, and the radioactivity counted as described above.

^{1}H NMR Spectroscopy—All of the spectra were recorded on a Bruker ARX 500 spectrometer equipped with a *z*-gradient unit. Peptide concentrations were ~2 mM. Each analog was examined in 95% H_2O, 5% D_2O, pH 3.0–3.5. The ^{1}H NMR experiments recorded were NOESY (18, 19) with a mixing time of 400 ms and TOCSY (20) with a mixing time of 65–120 ms. All of the spectra were recorded at 293 K and were run over 6024 Hz (500 MHz) with 4096 data points, 512 free induction decays, 16–80 scans, and a recycle delay of 1 s.

The solvent was suppressed using the WATERGATE sequence (21). Spectra were processed using XWINMR. Free induction decays were multiplied by a polynomial function and apodised using a 90° shifted sine-bell function in both dimensions prior to Fourier transformation. Base-line correction using a fifth order polynomial was applied. Chemical shift values were referenced internally to 2,2-dimethyl-2-silapetane-5-sulfonate at 0.00 ppm. The peptides were assigned according to the method of Wüthrich (22). Secondary Hα shifts were compared with the random coil shift values of Wishart *et al.* (23).

Materials—Desipramine hydrochloride, imipramine hydrochloride, mazindol, nisoxetine hydrochloride, (−)-norepinephrine bitartrate, and pargyline were obtained from Sigma. U-0521 and GBR-12909 dihydrochloride were from Biomol (Plymouth Meeting, PA). *l*-[7-^{3}H]Norepinephrine (specific activity, 14.9 Ci/mmol), 5-[1,2-^{3}H(*N*)]hydroxytryptamine creatinine sulfate ([^{3}H]serotonin) (specific activity, 24.0 Ci/mmol), 3,4-[7-^{3}H]dihydroxyphenylethylamine ([^{3}H]dopamine) (specific activity, 27.5 Ci/mmol), [^{3}H]mazindol (specific activity, 21 Ci/mmol), and [^{3}H]nisoxetine (specific activity, 80 Ci/mmol) were obtained from PerkinElmer Life Sciences. Protected Fmoc-amino acid derivatives were from Novabiochem or Auspep (Melbourne, Australia). The following side-chain protected amino acids were used: Cys(tBu), Asn(Trt), His(Trt), Hyp(tBu), Tyr(tBu), and Lys(Boc). Dimethylformamide, dichloromethane, diisopropylethylamine, and trifluoroacetic acid, were all of peptide synthesis grade supplied by Auspep. HBTU was Fluka 12804 supplied by Sigma. High performance liquid chromatography grade acetonitrile and methanol was supplied by Sigma. Resin used was Fmoc-rink amide resin supplied by Polymer Laboratories. Triisopropylsilane was from Aldrich.

Statistics and Data Analysis—Data are expressed as means ± S.E. of results obtained from 2 to 5 separate experiments. Student's two-tailed *t* test or, where appropriate, ANOVA with post hoc *t* tests performed by the Tukey method was used to evaluate the statistical significance of differences between groups. Values of $p < 0.05$ were considered significant. Curve fitting of concentration-response curves and radioligand binding data was performed by non-linear regression using individual data points with Prism 3.0 software for Macintosh (GraphPad, San Diego, CA). The equation of Cheng and Prusoff (24) was used to convert IC_{50} values to K_i values.

RESULTS

Effect of χ-MrIA on the Cellular Uptake of [^{3}H]Monoamines—COS-1 cells transfected with either the rat or human NET readily accumulated [^{3}H]norepinephrine, and nonspecific uptake of [^{3}H]norepinephrine was <2.5% of the total uptake. As shown in Fig. 1, the uptake of [^{3}H]norepinephrine via the rat and human NET was sensitive to inhibition by χ-MrIA with pIC_{50} values of 6.21 ± 0.02 (rat; $n = 3$) and 5.90 ± 0.03 (human; $n = 3$). χ-MrIA acted as a full inhibitor of the NET of both species. For DAT- and SERT-transfected cells, nonspecific uptake represented <6% of the total [^{3}H]norepinephrine accumulation. In the presence of χ-MrIA (100 μM), the rate of uptake of [^{3}H]dopamine by the human DAT and [^{3}H]serotonin by the human SERT was not significantly altered (Fig. 1*B*).

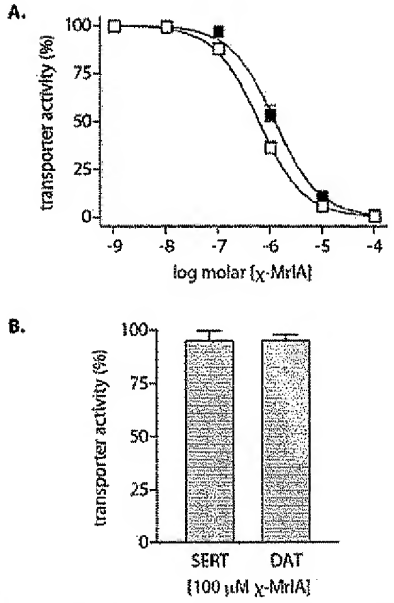

FIG. 1. **Effect of χ-MrIA on the activity of expressed monoamine transporters.** *A*, the rate of specific uptake of [^{3}H]norepinephrine into COS-1 cells transiently transfected with the rat NET (□) or human NET (■) was measured as described in the presence of the indicated concentrations of χ-MrIA. *B*, the rate of specific uptake of [^{3}H]5-HT and [^{3}H]dopamine by human SERT- and human DAT-transfected COS-1 cells, respectively, was determined in the presence of 100 μM χ-MrIA. Each data set was normalized to 100% activity for transport occurring in the absence of χ-MrIA. Specific uptake was defined by that which was sensitive to desipramine (10^{-4} M) for the rat and human NET, imipramine (10^{-6} M) for the human SERT, and GBR-12909 (10^{-6} M) for the human DAT. *Symbols* and *bars* represent the mean ± S.E. of four experiments performed in duplicate.

Effect of χ-MrIA on the Binding of Classical NET Inhibitors—χ-MrIA inhibited the binding of [^{3}H]nisoxetine to the membranes of cells expressing the rat and human NET (Fig. 2*A*). The IC_{50} for inhibition was 500 nM (pK_i = 6.6 ± 0.05) at the rat NET and 1.7 μM (pK_i = 6.0 ± 0.04) at the human NET. [^{3}H]Mazindol binding to the expressed transporters was also sensitive to χ-MrIA (Fig. 2*B*). χ-MrIA inhibited binding with an IC_{50} of 1.9 μM (pK_i = 6.4 ± 0.03) at the rat NET and 4.0 μM (pK_i = 5.9 ± 0.04) at the human NET. Nonspecific binding represented ~3% of the total binding in all of the experiments, and χ-MrIA acted as a full inhibitor of [^{3}H]nisoxetine and [^{3}H]mazindol binding.

Saturation analysis was used to characterize the nature of the inhibition caused by χ-MrIA (Fig. 3). In the absence of χ-MrIA, [^{3}H]nisoxetine bound to rat NET membranes with a K_d of 4.2 ± 0.5 nM and a B_{max} of 42 ± 1.6 pmol/mg protein. The K_d was increased to 21 ± 3.6 nM in the presence of 2 μM χ-MrIA with no significant change in the value of the B_{max} (45 ± 4.2 pmol/mg protein). For [^{3}H]mazindol binding, the K_d was 1.0 ± 0.1 nM and the B_{max} was 40 ± 0.3 pmol/mg protein in control experiments. In the presence of χ-MrIA (20 μM), the K_d (35 ± 1.5 nM) but not the B_{max} (41 ± 0.6 pmol/mg protein) was significantly altered. χ-MrIA (10^{-6} M) did not affect the dissociation rate of [^{3}H]nisoxetine from the expressed rat NET (data not shown).

Desipramine, nisoxetine, and χ-MrIA reduced the binding of [^{3}H]nisoxetine to rat brain homogenates in a concentration-dependent manner (Fig. 4). *F* tests comparing the fit of the binding data to a model of one-site competition, two-site competition, or a sigmoidal curve with a variable slope indicated that the simpler one-site competition model was preferred and more complicated models did not significantly improve the fit ($p >$ 0.2 for each of the comparisons). The IC_{50} values for the inhibition were 1.1 nM (pIC_{50} = 8.9 ± 0.06) for desipramine, 6.2 nM (pIC_{50} = 8.2 ± 0.07) for nisoxetine, and 5.7 μM (pIC_{50} = 5.2 ± 0.22) for χ-MrIA. While desipramine and unlabeled nisoxetine inhibited the [^{3}H]nisoxetine binding to the same extent (nonspecific binding of ~43%), the estimated maximum extent of inhibition produced by χ-MrIA was significantly less ($p <$

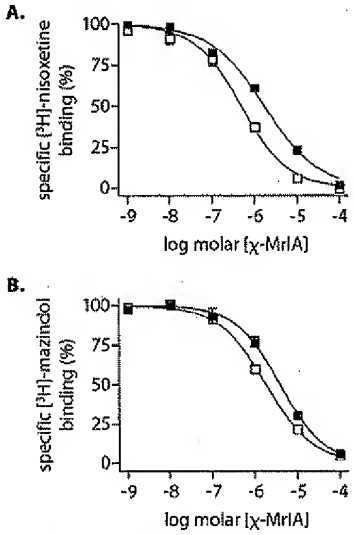

FIG. 2. **Inhibition of the binding of classical inhibitors to the expressed NET by χ-MrIA.** The specific binding of [^{3}H]nisoxetine (*A*) and [^{3}H]mazindol (*B*) to the membranes of COS-7 cells that had been transiently transfected with the rat NET (■) or the human NET (□) was examined in the presence of the indicated concentrations of χ-MrIA. Radioligand binding determined in reactions without χ-MrIA was used to define 100% binding. Nonspecific binding was defined as that occurring in the presence of 100 μM desipramine. *Symbols* represent the mean ± S.E. of three experiments performed in triplicate.

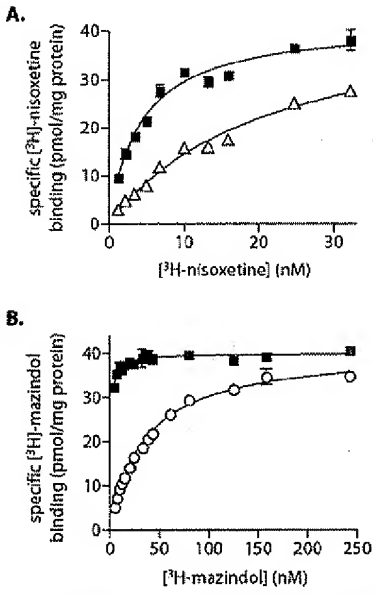

FIG. 3. **Effect of χ-MrIA on the saturability of binding of classical inhibitors to the expressed rat NET.** The specific binding of increasing concentrations of [^{3}H]nisoxetine (*A*) and [^{3}H]mazindol (*B*) to membranes from COS-7 cells transiently transfected with the rat NET was measured in the absence (■) and presence of χ-MrIA (△, 2 μM; ○, 20 μM). Nonspecific binding was defined as that occurring in the presence of 100 μM desipramine. *Symbols* represent the mean ± S.E. of three experiments performed in triplicate.

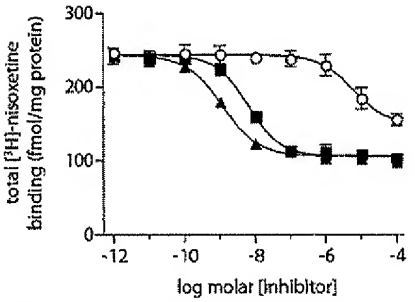

FIG. 4. **Inhibition of [^{3}H]nisoxetine binding to rat brain.** The effect of increasing concentrations of desipramine (▲), unlabeled nisoxetine (■), and χ-MrIA (○) on the total binding of [^{3}H]nisoxetine to rat brain homogenates (20 μg of protein) was examined as described. *Symbols* represent the mean ± S.E. of three experiments performed in triplicate.

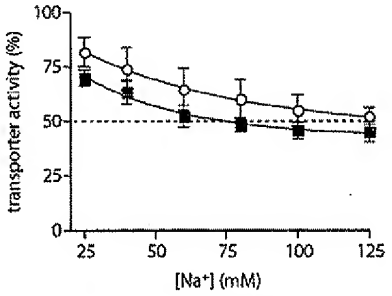

FIG. 5. **Sodium dependence of the effectiveness of NET inhibitors to block uptake.** COS-1 cells transiently transfected with the human NET were exposed to [^{3}H]norepinephrine (100 nM) contained in buffer in which various amounts of Na^+ had been isotonically replaced with Li^+. Specific uptake of [^{3}H]norepinephrine occurring in the presence of desipramine (○, 4.05 nM) and χ-MrIA (■, 1.26 μM) was expressed as a percentage of the uptake occurring in the absence of the inhibitors at each extracellular Na^+ concentration tested. Nonspecific uptake was defined by that which was not sensitive to 100 μM desipramine. *Symbols* represent the mean ± S.E. of three experiments performed in duplicate.

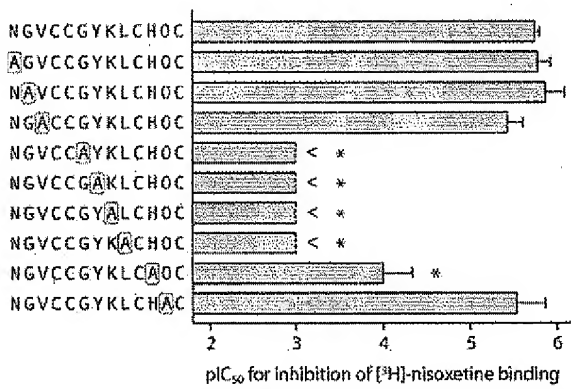

FIG. 6. **Alanine scan of χ-MrIA.** A series of analogs of χ-MrIA in which non-cysteine residues were systematically replaced with alanine was assayed for inhibition of [^{3}H]nisoxetine binding to the expressed human NET, and their potency was compared with χ-MrIA (*top bar*). The potency of analogs with bars marked "<" are estimates based on the lack of any inhibition being detected at concentrations up to 100 μM. *, $p < 0.05$ compared with χ-MrIA. *Bars* represent the mean ± S.E. of 2–5 experiments performed in triplicate.

0.001) with ~32% of the nisoxetine- and desipramine-sensitive binding found to be insensitive to χ-MrIA.

Sodium dependence of NET inhibition—The rate of uptake of [^{3}H]norepinephrine by cells transfected with the human NET slowed substantially as the concentration of Na^+ in the transport buffer was reduced. At the lowest Na^+ concentration examined (25 mM), the rate of [^{3}H]norepinephrine accumulation was approximately half of that observed at 125 mM Na^+ (data not shown). Concentrations of desipramine and χ-MrIA that inhibited transport by 50% in assays where the buffer contained 125 mM Na^+ (4.05 nM and 1.26 μM, respectively) were found to inhibit a progressively smaller proportion of the uptake in buffer containing less Na^+ (Fig. 5).

Effect of Residue Replacement on the Potency of χ-MrIA—Nine analogs of χ-MrIA in which the non-cysteine residues were systematically replaced with alanine were assayed for inhibition of [^{3}H]nisoxetine binding to the expressed human NET, and their potency was compared (Fig. 6). The analogs with substitutions at N-terminal residues outside of the cysteine-bracketed loops ([N1A]MrIA, [G2A]MrIA, and [V3A]MrIA) displayed no significant change in potency compared with χ-MrIA. The replacement of any of the residues located in the first cysteine-bracketed loop, in contrast, had a severe impact on potency. No inhibition was observed with these analogs ([G6A]MrIA, [Y7A]MrIA, [K8A]MrIA, and [L9A]MrIA) at 100 μM, the highest concentration tested. Assuming that the Hill slope parameter for their inhibition remains unchanged compared with χ-MrIA, the IC_{50} concentrations of these peptides will be at least an order of magnitude greater still, yielding a conservative estimate of 10^{-3} M. Alanine substitution of the first residue of the second cysteine-bracketed loop (analog [H11A]MrIA) caused a ~60-fold reduction in potency. Replacement of the other residue in this loop (analog [O12A]MrIA) did not have a significant effect on potency. Two further analogs were assayed to investigate the effect of replacement with residues other than alanine at positions 7 and 8. The potency of [Y7F]MrIA was ~3.8-fold lower (pIC_{50} = 5.2 ± 0.08) than χ-MrIA, and the potency of [K8R]MrIA was ~6.8-fold lower (pIC_{50} = 4.9 ± 0.10) than χ-MrIA.

Structural Effects of Alanine Substitutions—1D, TOCSY, and NOESY ^{1}H NMR spectra of χ-MrIA and analogs were recorded at 500 MHz and subsequently assigned using the sequential assignment protocol (22). Secondary chemical shifts, *i.e.* Hα chemical shifts compared with random coil values (25), are a sensitive measure of backbone conformation (26–28) and can provide an indication whether the overall global fold of a series of a peptide is maintained (29). For a series of structurally related peptides, secondary Hα chemical shifts can be used to identify the location but not the nature of local changes in conformation (29). Secondary Hα chemical shifts were used in the first instance to compare χ-MrIA with its alanine-substituted analogs (Fig. 7). The results indicate that the overall global fold of the χ-MrIA analogs used in this study are conserved compared with native χ-MrIA with the exception of [G6A]MrIA where the overall fold of the peptide appears different. Small local changes are observed for [K8A]MrIA and [H11A]MrIA at the site of the altered residue. For [Y7A]MrIA, a small change in the secondary Hα chemical shift is seen at Lys-8. This is not surprising as Tyr-7 is a relatively large residue that, relative to Ala-7, could influence the chemical environment of Lys-8 and hence differentially influence its Hα chemical shift. In the case of [G6A]MrIA, a comparison of its secondary Hα chemical shifts with χ-MrIA indicates that replacement of this residue causes a significant structural perturbation. Interestingly, introduction of a stereocenter through substitution of Gly-6 with an alanine appears to alter the structural rigidity of [G6A]MrIA. This enhanced structural rigidity for [G6A]MrIA is supported by changes in secondary Hβ shifts for residue Cys-5 where the two Cys-5 β-protons are well separated in [G6A]MrIA. In contrast, the other χ-MrIA analogs investigated in this study all display degenerate β-pro-

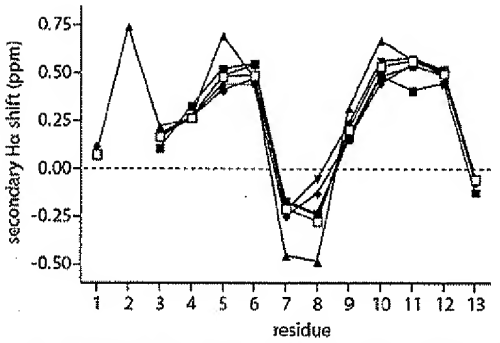

FIG. 7. **^{1}H NMR spectroscopy of χ-MrIA and its alanine-substituted analogs.** Secondary Hα chemical shifts (ppm) for MrIA analogs show the similarity in global fold between native χ-MrIA (□) and the [Y7A]- (▼), [K8A]- (♦), [L9A]- (●), and [H11A]- (■) analogs and structural perturbation for [G6A]-MrIA (▼). Secondary Hα shifts were derived from TOCSY spectra recorded at 500 MHz and 293 K at a peptide concentration of ~2 mM.

tons (data not shown). The relative position of the side chains of Tyr-7, Lys-8, Leu-9, and His-11 of χ-MrIB (equivalent to χ-MrIA in structure (7)) are shown in Fig. 8.

DISCUSSION

The aim of the present study was to investigate the influence that the transporter identity, the co-substrate Na^+, and individual residues of χ-MrIA have on the ability of the conopeptide to inhibit monoamine transporters. Whether χ-MrIA acted through a site on the NET that was distinct from the classical inhibitors of the NET was also examined. χ-MrIA inhibited uptake by the NET of both species studied and was found for expressed transporters to act with twice the potency at the rat over the human isoform. The amino acid sequence homology between the NETs of the two species is 93% (14). The NET is related to the transporters for the other monoamine neurotransmitters, dopamine and serotonin. The amino acid identity between the human NET and the human DAT is 66% (15), and between the human NET and human SERT, the homology is 43% (16). Because a substantial number of inhibitors of the NET have relatively low specificity and also target the DAT, the SERT, or both (30), the effect of χ-MrIA on transport by the human DAT and SERT was of interest. Our finding that χ-MrIA at a concentration of 100 μM, which is ~90 times the IC_{50} for inhibition of [^{3}H]norepinephrine transport by the NET, did not significantly affect the operation of either the DAT or the SERT to take up their ^{3}H-substrates demonstrates that χ-MrIA acts with a very high degree of specificity. It also demonstrates that the action of χ-MrIA is not an indirect one to inhibit transport by a more general mechanism such as disturbing the transmembrane ionic gradients that are coupled to transport because this would influence the activity of all three transporters (31, 32). Unlike χ-MrIA, the peptidic transporter inhibitors identified by Koppel *et al.* (33) and Rothman *et al.* (34) from combinatorial chemistry libraries do not discriminate between individual members of the monoamine neurotransmitter transporter family, targeting both the DAT and the SERT with IC_{50} values in the micromolar range. Their activity at the NET has not been reported.

We previously showed that the inhibition of the NET by χ-MrIA is reversible and non-competitive with respect to substrate (7). This non-competitive mode of action distinguishes χ-MrIA from classical NET inhibitors including cocaine, mazindol, nisoxetine, and other antidepressants, which are competitive inhibitors of [^{3}H]norepinephrine uptake (2, 35–37). Here, radioligand binding experiments have revealed that χ-MrIA acts competitively with respect to [^{3}H]nisoxetine and [^{3}H]mazindol indicated by the effect of χ-MrIA to increase the apparent K_d for the binding of the radioligands to the expressed rat NET without reducing the value of the B_{max}. Consistent with functional experiments, χ-MrIA is a somewhat more potent inhibitor at the rat NET than the human NET. The identification of various residues of the NET that affect substrate and antidepressant affinity either jointly or separately (38–42) indicates that the antidepressant binding site partially overlaps the substrate binding site. Our finding that χ-MrIA competes with nisoxetine and mazindol but not norepinephrine implies that the χ-MrIA binding site is wholly distinct from the substrate binding site but shares some identity with the antidepressant binding site. The results from recent site-directed mutagenesis experiments with the human NET (43), in which the affinities of some of the mutant transporters for χ-MrIA, desipramine, and cocaine were found to change in parallel and others change selectively for the different ligands, provide further support for a partial overlap among the χ-MrIA, desipramine, and cocaine binding sites.

Although the potency of χ-MrIA for inhibition of uptake and radioligand binding to the expressed rat NET observed here closely matches its reported potency for potentiating noradrenergic contractions in the isolated rat vas deferens (430 nM; 7), we found that the potency of χ-MrIA for inhibition of the binding of [^{3}H]nisoxetine to rat brain was an order of magnitude lower. Given its lower potency in the rat brain, its only partial inhibitory effect and the modest degree of assumed specific (*i.e.* nisoxetine- or desipramine-sensitive) binding in the assay, it is perhaps not surprising that McIntosh *et al.* (9) did not detect any effect of χ-MrIA (10 μM) in their NET binding assay using conditions somewhat similar to those used here. A possible reason for the only partial inhibition of the specific [^{3}H]nisoxetine binding by χ-MrIA is the additional binding of desipramine and nisoxetine to sites in the rat brain other than the NET such as α_1-adrenoceptors (44) or the SERT (30), which are not also targeted by χ-MrIA. Alternatively, the classical NET inhibitors may bind at multiple sites on the NET in a manner reminiscent of the interaction of the cocaine analog RTI-55 and the SERT (45) with χ-MrIA blocking only a subset of these sites. Our finding that χ-MrIA acts as a full inhibitor of the desipramine-sensitive [^{3}H]nisoxetine binding to rat NET-transfected cell membranes (Fig. 2*A*) discounts this hypothesis or at least reflects a difference in the presentation of the NET in the membranes of native tissues and transfected cells or even the existence of NET subtypes in the rat. The existence of such subtypes could explain the unexpected reduction in the potency of χ-MrIA observed in the brain binding assay.

Norepinephrine transport by the NET has been shown to be dependent on Na^+, reflecting the co-transport of Na^+ with the substrate (46). The reduced transport activity caused by lowering of the extracellular Na^+ concentration is mediated through an increase in the apparent K_m for norepinephrine and a reduction in the V_{max}. Extracellular Na^+ not only affects the affinity of the transporter for substrate but also its affinity for inhibitors. It has previously been shown that desipramine and other antidepressants become less effective inhibitors of uptake with reduced extracellular Na^+ (47), an observation confirmed in this study. χ-MrIA demonstrates the same pattern of Na^+ dependence. These findings may signify that desipramine and χ-MrIA target the outward-facing (substrate-accessible) configuration of the transporter whose adoption is promoted by extracellular Na^+ (31). The Na^+ dependence of the inhibitory action of χ-MrIA stands in contrast to that of cocaine, another natural product that inhibits the NET. Cocaine competes with Na^+ for binding to the NET, becoming a more potent inhibitor of transport as extracellular Na^+ decreases (47).

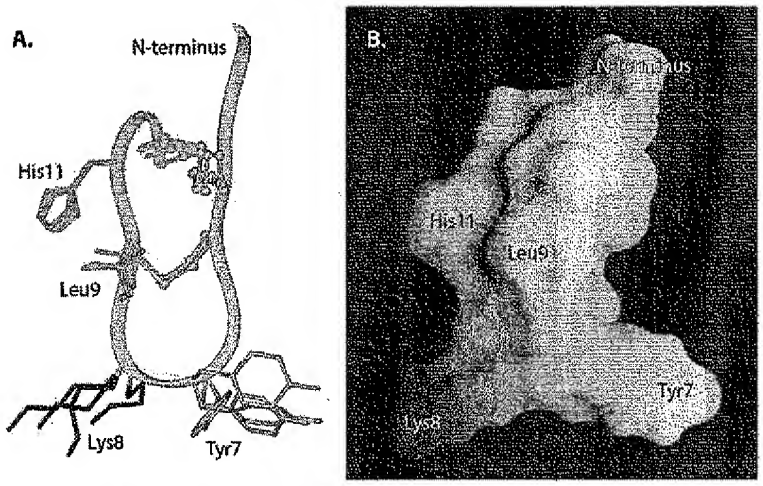

FIG. 8. **Pharmacophore model of χ-MrIA.** *A*, ribbon representation of χ-MrIA with residues determined to be important for interaction with the NET indicated as follows: Tyr-7 (*pink*), Lys-8 (*blue*), Leu-9, and His-11 (*red*). Disulfide connectivity is shown in *orange*. *B*, electrostatic surface of χ-MrIA with residues 7–9 and 11 and the N terminus labeled. Positively charged surface is shown in *blue*, and hydrophobic surface shown in *white*. The model of χ-MrIA was generated in Insight and was based on the solution structure of χ-MrIB (7) (Protein Data Bank accession number 1IEO) using residue replacement of Val-1 in χ-MrIB to Asn-1 (the corresponding residue in χ-MrIA). The ribbon representation was generated using Insight 2000.1 (50), and the electrostatic surface was generated using GRASP (51) on a Silicon Graphics Octane computer.

The three-dimensional structure of χ-MrIA has not been determined but appears very similar to that of χ-MrIB (7), a conopeptide whose sequence differs by only a single residue at the N terminus and that displays very similar pharmacology to χ-MrIA. Similar to the majority of conopeptides, χ-MrIA contains multiple cysteine residues that are linked by intramolecular disulfide bonds. These bonds act to bring the cysteine pairs into close proximity in the core of the peptide with residues in the intercysteine regions exposed as loops. Alanine scanning reveals a critical role for residues in the first and largest of χ-MrIA's two cysteine-bracketed loops in contributing to the activity of the peptide at the NET. Substitution of any of the residues in this region with alanine results in a loss of potency predicted to be in excess of ~600-fold. With the exception of the replacement of Gly-6, the alanine substitutions did not affect the structure of the peptide backbone to any great degree. Tyr-7, Lys-8, Leu-9, and to a lesser extent His-11 therefore seem likely to directly interact with the transporter, whereas Gly-6 probably plays a structural role to allow the correct orientation of the other residues in the loop for better NET binding. The involvement of tyrosine and lysine residues in the high affinity interaction of other peptide toxins with their targets has been reported previously (48, 49), warranting further investigation into χ-MrIA's use of these residues as high affinity binding determinants in experiments with additional analogs. Phenylalanine was found to be able to largely substitute for Tyr-7, indicating that the hydroxyl group of the tyrosine residue is not of critical importance for binding. This stands in contrast to its role in the interaction of the ω-conotoxins with the $Ca_v2.3$ channel (48). The replacement of arginine with lysine at position 8 had a relatively small detrimental effect on potency, showing that the side-chain charge may be a key determinant for binding at this position and that length is less influential.

Examination of the relative positions of the key binding determinants in χ-MrIA (Fig. 8) reveals a highly exposed Lys-8 flanked by three hydrophobic residues to form the pharmacophore. Given its exposed position, it is possible that Lys-8 could direct the binding into a pore, perhaps reminiscent of how toxins such as charybdotoxin block the movement of K^+ ions through voltage-dependent channels (49). If this is indeed correct as suggested by Bryan-Lluka *et al.* (43), χ-conopeptides might be useful molecular calipers to probe the size of the norepinephrine permeation pathway. The majority of transporter residues that have been found to influence tricyclic antidepressant binding lie in the predicted transmembrane domains of the NET (*e.g.* 41, 52, 53). That the binding site of the antidepressants overlaps with that of norepinephrine, yet the binding site of χ-MrIA does not, is consistent with the χ-conopeptides acting to inhibit transport by blocking the access of norepinephrine to the substrate permeation pathway by binding less deeply in the pore of the transporter than do the classical small molecule inhibitors.

In summary, this study has shown that inhibition of monoamine transport by χ-MrIA is confined to that mediated by the NET. Accordingly, the χ-MrIA binding site on the NET seems likely to consist, at least partly, of residues that are not conserved between the NET and either the DAT or the SERT. Because χ-MrIA acts non-competitively with respect to norepinephrine yet competitively with the classical NET inhibitors nisoxetine and mazindol, which themselves are competitive inhibitors of norepinephrine uptake, the binding site of χ-MrIA is predicted to be distinct from the substrate binding site but to share some commonality with the antidepressant binding site. The Na^+ dependence exhibited by χ-MrIA indicates some similarity in its interaction with the NET to that of the antidepressants. Specific residues in loop one of χ-MrIA have been identified that directly interact with the NET or are important for the maintenance of a suitable peptide structure capable of recognizing the transporter. Furthermore, elucidation of how the χ-MrIA peptide binds to and inhibits the NET will reveal important structural and mechanistic information regarding the monoamine transporters which, at present, are poorly understood.

Acknowledgments—We thank Lesley Bryan-Lluka (The University of Queensland) for kindly providing the rat NET vector, Marc Caron (Duke University Medical Center) for the human DAT vector, and Randy Blakely (Vanderbilt University) for the human SERT vector.

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